U.S. Food and Drug Administration

Animal Health Literacy

CVM's OR Develops New PCR-Based Method for Testing Animal Feed

The Office of Research (OR) of the U.S. Food and Drug Administration's Center for Veterinary Medicine (CVM) recently developed a new method for testing animal feed for prohibited materials. The method relies on polymerase chain reaction (PCR), a molecular technique that amplifies small amounts of genetic material (DNA or RNA) to produce larger amounts for analysis. Once the new PCR-based method is routinely used, it will enhance FDA's ability to make sure animal feed is safe and free of prohibited materials that may spread the agent thought to cause bovine spongiform encephalopathy (BSE).

BSE is a fatal disease that causes progressive degeneration of the central nervous system (brain and spinal cord) in cattle. BSE was first detected in the United Kingdom (U.K.) in 1986. Studies quickly established an association between outbreaks of BSE and the use of cattle feed containing protein from cattle and other ruminants, such as sheep and goats. In 1988, the U.K. issued the world's first feed ban prohibiting ruminant meat-and-bone meal from being fed to cattle and other ruminants.

On June 5, 1997, FDA issued a similar feed ban that prohibited most mammalian protein from being used to make animal feed for ruminants (ref 1). In April 2008, FDA strengthened the feed ban by prohibiting high-risk materials from being used to make all animal feed, including pet food. High-risk materials are those materials from cattle that have the highest chance of carrying the agent thought to cause BSE, such as the brains and spinal cords from cattle that are 30 months of age or older.

Testing Feed Samples: The Current Process

To make sure that animal feed manufacturers comply with the feed ban, FDA tests feed samples for prohibited materials. Feed samples are typically collected by field investigators in the Office of Regulatory Affairs (ORA), FDA's investigative arm. The samples are analyzed by feed microscopy, a technique that uses a microscope to visually identify the components in the sample. Samples that test positive for a prohibited mammalian protein by feed microscopy then undergo PCR testing to confirm the positive result.

In 2001, FDA validated a PCR-based method capable of detecting mammalian protein in animal feed (ref 2). OR's feed analysts used the 2001 method for several years when they were asked by ORA to confirm the presence of prohibited materials in animal feed, although the method was never used by field investigators in ORA. In 2006, OR validated an improved version of the 2001 PCR-based method. After extensive hands-on training, ORA's field investigators began using the 2006 method (ref 3) to confirm positive feed microscopy results.

The Traditional PCR-Based Method

Both the 2001 and 2006 PCR-based methods rely on agarose gel electrophoresis. Gel electrophoresis is a technique that separates materials by size using an electric field applied to a gel. In agarose gel electrophoresis, the gel is made from agarose, a gelatinous substance derived from seaweed.

The DNA from an animal feed sample is extracted and amplified using PCR. A small amount of the DNA is then placed in a pre-cut hole, or "well," at the top of the agarose gel. When an electric field is applied to the gel, the DNA pieces in the sample move towards the bottom of the gel. The rate of speed depends on size. Smaller pieces move faster than larger pieces. The DNA pieces separate into distinct bands based on size, where one band represents DNA pieces of the same size. The size of a piece of DNA is determined by the number of base pairs (abbreviated "bp") it contains. The higher the bp number, the more base pairs make up that particular piece of DNA; and the more base pairs, the larger the piece of DNA.

When a fluorescent dye is added to the gel, the bands fluoresce under ultraviolet (UV) light and a photograph is taken which shows the location of the fluorescing bands within the gel (see Figure 1). Based on the location of the bands, the size of the DNA pieces can be determined.

Because the DNA pieces in a sample move in a straight line from the top to the bottom of the gel, the sample is said to be in a "lane." One gel usually has several lanes for analyzing multiple samples at the same time.

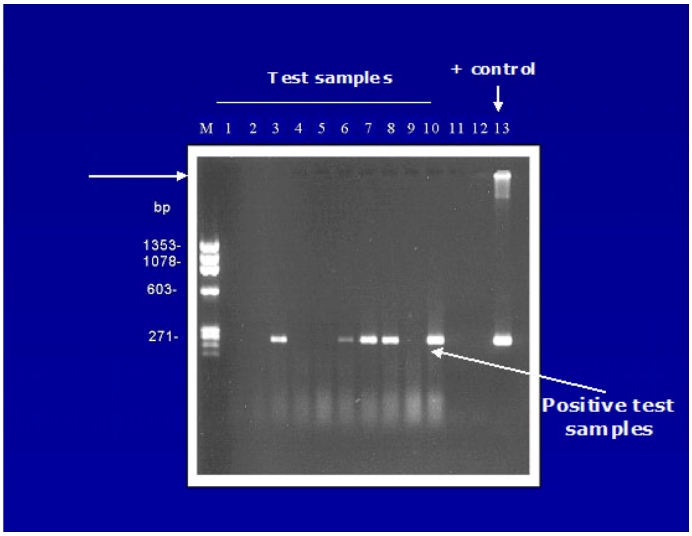

Figure 1. Photographic Image of the Traditional PCR-Based Method

The arrow at the top left of the image is pointing to the wells which appear as dark rectangles at the top of the gel. Lane "M" contains a set of standard DNA pieces with known sizes. Lanes 1 through 10 contain the feed samples that are to be tested for prohibited materials. Lanes 11 and 12 are the negative control samples and do not contain any prohibited materials. Lane 13 is the positive control sample and contains

cattle protein, a prohibited material. A fluorescing band at the 271 bp location means that the animal feed sample likely contains prohibited cattle protein.

The 2006 Method: Limitations

PCR-based methods that rely on UV light for visualizing the bands commonly have problems. Most of the problems encountered during OR's validation testing of the 2006 method had to do with the process of agarose gel electrophoresis or interpreting the resulting photograph (ref 3). For example, when a group of feed samples containing negative, slightly positive, and highly positive samples is analyzed, the resulting photograph is often a hard-to-read compromise between the lowest-intensity bands, which are faint to undetectable, and the highest-intensity bands, which are so bright that they hide nearby lanes (ref 4). Also, the instrument that takes the photograph of the gel is usually set to automatically focus on the brightest bands, causing bands of lesser intensity to be barely visible or not visible at all (see Figure 2). Another problem with the 2006 method is that it takes at least 8 hours to analyze a sample.

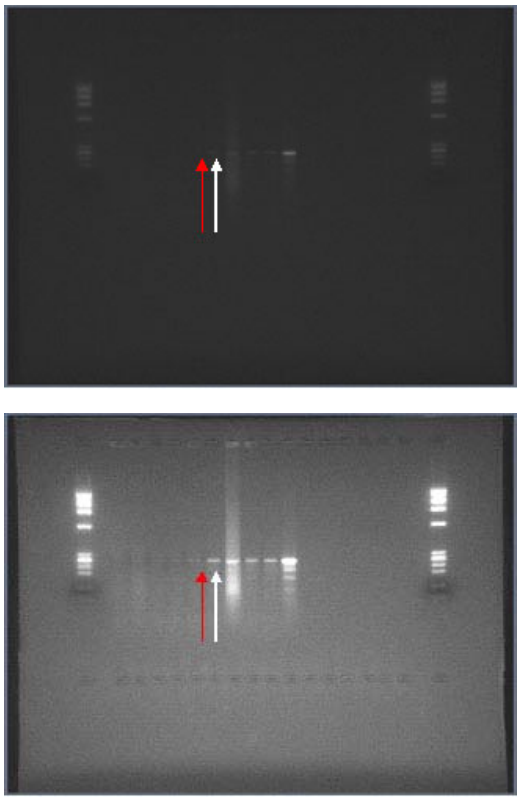

Figure 2. Photographic Example of the Limitations of the 2006 Method

The upper photograph was taken using the automatic settings for the instrument that takes the photograph of the agarose gel. The instrument focused on the brightest bands in the gel, causing one positive feed sample to barely show up (light arrow on the right) and a second positive sample to not show up at all (dark arrow on the left). The lower photograph is the same gel after the settings were manually adjusted. Now, both positive samples can be clearly seen.

The 2009 Method: Goals and Solutions

OR's first goal was to develop a faster, simpler method based on real-time PCR. In real-time PCR, a fluorescent dye is used to tell how much DNA is being produced during the reaction. The fluorescent dye binds to the DNA and the bound DNA fluoresces and emits a light signal that is detected by the PCR instrument (see Figure 3).

OR's second goal was to evaluate the new method using strict in-house testing requirements and a peer verification trial.

Because there are no set federal guidelines to evaluate methods to detect prohibited materials in animal feed, OR had to develop its own objective criteria (called acceptance criteria) to assess the new real-time PCR-based method. The acceptance criteria used in the assessment had previously been used by OR to evaluate four kits currently on the market to detect prohibited mammalian protein in animal feed (refs 5 and 6). OR also used a statistical approach to determine if the acceptance criteria were met.

An important criterion was the ability of the new method to detect mammalian protein when present in animal feed at a concentration of at least 0.1%. This value was chosen because (a) 0.1% was the concentration used in the validation trials for the 2001 and 2006 PCR-based methods (refs 2 and 3); and (b) 0.1% is the concentration that is normally detected by feed microscopy (ref 7).

The new real-time PCR-based method met strict requirements for sensitivity (ability to detect true positive samples), selectivity (ability to detect true negative samples), and specificity (ability to detect only the targeted animal species). It also met strict requirements for ruggedness and real-time platform. The ruggedness test determines how well the method tolerates small changes to its set operating limits and measures the method's reliability under normal use. The real-time platform test assesses how well the PCR portion of the method works using different laboratory instruments.

After strict in-house testing, the new method underwent a peer verification trial to assess how well it worked when used by other laboratories and if the instructions on how to use it were clear. Two outside laboratories participated in the peer verification trial. The results showed that the method had 100% specificity in identifying three types of prohibited materials, cattle meat-and-bone meal, lamb meal, and goat meat meal. There was only a 0.6% rate of false positive results. The peer verification trial proved that the new real-time PCR-based method can easily and reliably be used by other laboratories.

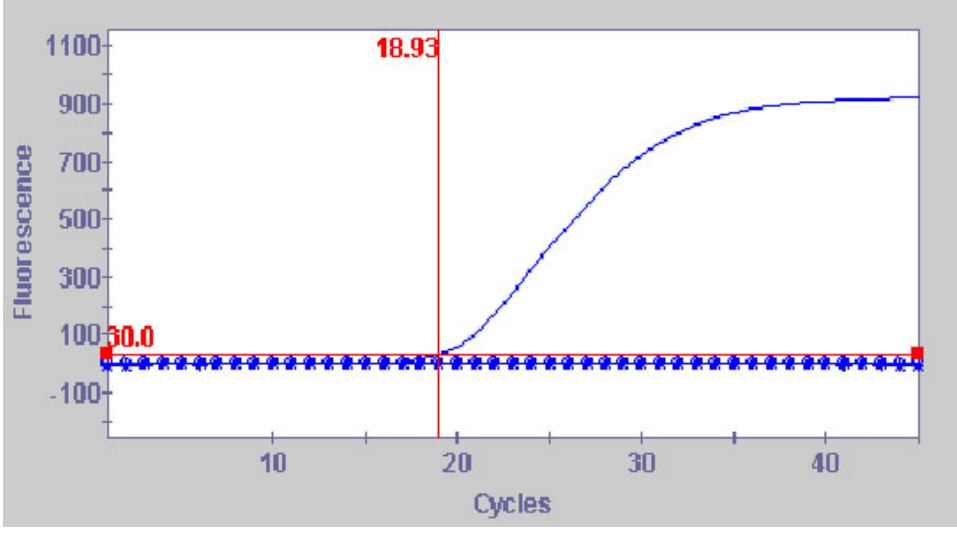

Figure 3. Computer Image of the New Real-Time PCR-Based Method

The level of fluorescence is shown on the y-axis and the cycles of PCR amplification is shown on the x-axis. The baseline level of fluorescence is 30

fluorescence units (horizontal solid line). The negative control sample (horizontal dotted line) does not contain any prohibited materials and fluoresces below the baseline level. A feed sample that contains a prohibited material fluoresces above the baseline level. The point at which the sample's fluorescence exceeds the baseline level is called the inflection point (vertical line). The inflection point is also called the Cycle Threshold (Ct) value. In the above image, the Ct value is 18.83. The lower the Ct value, the higher the concentration of DNA from prohibited materials in the sample.

The 2009 Method: Advantages

In less than 2.5 hours, the new real-time PCR-based method can detect processed materials from cattle, sheep, and goats, as well as a select set of processed materials from chickens, turkeys, and geese (ref 8). The method can also detect animal materials that have been processed in both North America and the European Union (E.U.). The processing conditions in the E.U. are very different from those in North America, resulting in meat-and-bone meals with different characteristics. Because it can detect meat-and-bone meals processed in both North America and the E.U., the 2009 method is more useful and versatile than the 2006 method.

Besides being faster and more versatile, another advantage of the new real-time PCR-based method is that all the components are available as a pair of commercial kits that are made under strict quality controls. One kit contains the reagents needed to extract the DNA from the feed sample for amplification by PCR. The second kit contains the reagents needed to perform the PCR test. Having all the necessary reagents available for laboratories in ready-to-use kits that have already been examined for quality control further reduces the time needed to analyze a sample.

Future Plans

The new 2009 real-time PCR-based method will become FDA's "one-stop shop" for testing animal feed. It will replace feed microscopy as FDA's method of choice for screening animal feed for prohibited materials. It will also be used to confirm the presence of prohibited materials in animal feed, replacing the 2006 PCR-based method.

OR is working with CVM's Office of Surveillance and Compliance on a plan to replace the 2006 method with the improved 2009 method. An integral part of the plan includes training sessions for ORA's federal field investigators at OR's research laboratories in Laurel, Maryland. State field investigators will also be invited to the training sessions. So far, OR has conducted three 3-day sessions to provide extensive hands-on training in this new method. Fourteen federal field investigators from ORA and 20 state field investigators from various states participated.

Once it fully replaces both feed microscopy and the 2006 PCR-based method, the new 2009 real-time PCR-based method will increase FDA's ability to detect prohibited mammalian protein in animal feed and prevent BSE in U.S. cattle. This strengthens the FDA's mission to protect animal and public health by keeping the food supply safe for both animals and people.

References

1. Federal Register, vol. 62, no. 108, p. 30976. June 5, 1997. Substances prohibited from use in animal food or feed: Animal proteins prohibited in ruminant feed. Final Rule. 21 CFR 589.2000.

2. Myers, M.J., S.L. Friedman, D.E. Farrell, D.A. Dove-Pettit, M.F. Bucker, S. Kelly, S. Madzo, W. Campbell, R.F. Wang, D. Paine, and C.E. Cerniglia. 2001. Validation of a polymerase chain reaction method for the detection of rendered bovine-derived materials in feedstuffs. *J. Food Prot*. 64:564-566.

3. Myers M.J., H.F. Yancy, J. Derr, J. Sedwick, M. Araneta, J. Wetherington, G. Mowlds, K. Riter, H. Lin, J. Armour, H. Koch, P. Istafanos, Y. Shen, R. Younkins, D. Pinero, F. Jackson, D. Farmer, W.M. Kiessling, and L.A.D Hoostelaere. 2006. Validation of a PCR-based method for the detection of various rendered materials in feedstuffs using a forensic DNA extraction kit. *J. Food Prot*. 69:205-210.

4. Yancy, H.F., A. Mohla, D.E. Farrell, and M.J. Myers. Evaluation of a rapid PCR-based method for detection of animal materials. 2005. *J. Food Prot*. 68:2651-2355.

5. Myers M.J., H.F. Yancy, D.E. Farrell, J.D. Washington, and R.A. Frobish. Evaluation of two commercial lateral-flow test kits for detection of animal proteins in animal feed. 2005. *J. Food Prot*. 68:2656-2664.

6. Myers M.J., H.F. Yancy, D.E. Farrell, J.D. Washington, C.M. Deavers, and R.A. Frobish. Assessment of Two Enzyme-Linked Immunosorbent Assay Tests Marketed for Detection of Ruminant Proteins in Finished Feed. 2007. *J. Food Prot.* 68:2656-2664.

7. Myers, M.J. Detecting animal tissues in feed and feed ingredients. Improving the Safety of Fresh Meat, J.N. Sofos, ed. 2005. Wood Head Publishing Ltd.: Cambridge, UK, pp. 55-79.

8. Yancy, H. F., J.D. Washington, L. Callahan, J.A. Mason, C.M. Deaver, D.E. Farrell, and M.J. Myers. 2009. Development, Evaluation, and Peer-Verification of a Rapid Real-Time PCR Method for the Detection of Animal Material. *J. Food Prot.* 72:2368-2374.